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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 10 April 2001 (10.04.01)	
International application No. PCT/GB00/02791	**Applicant's or agent's file reference** P022288WO
International filing date (day/month/year) 19 July 2000 (19.07.00)	**Priority date** (day/month/year) 19 July 1999 (19.07.99)
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1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

19 February 2001 (19.02.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Juan Cruz
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 January 2001 (25.01.2001)

PCT

(10) International Publication Number
WO 01/05823 A2

(51) International Patent Classification[7]: C07K 14/00

(21) International Application Number: PCT/GB00/02791

(22) International Filing Date: 19 July 2000 (19.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9916913.8 19 July 1999 (19.07.1999) GB

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/05823 A2

(54) Title: INHIBITOR PROTEINS

(57) Abstract: The present invention relates to novel protease inhibitor proteins that have been identified in ticks. These proteins may be used as components of vaccines, as inhibitors of mast cell tryptase, in detection of mast cells and in the isolation and purification of mast cell tryptase. The invention also relates to the control of diseases and injury caused by parasites in animals and humans and to the use of the proteins of the invention in the treatment of certain diseases and allergies.

INHIBITOR PROTEINS

The present invention relates to novel proteins that have been identified in ticks. These proteins may be used a components of vaccines, as inhibitors of mast cell tryptase (hereafter referred to as MCT), in the detection of mast cells and in the isolation and purification of MCT. The invention also relates to the control of diseases and injury caused by parasites in animals and humans and to the use of the proteins of the invention in the treatment of diseases and allergies.

All documents mentioned in the text and listed at the end of this description are incorporated herein by reference.

Human MCT is an endoprotease that is stored in the secretory granules of mast cells and, upon activation, is released from the mast cells as a tetramer that is stabilised by heparin. Removal of heparin leads to the dissociation of the tryptase complex into enzymatically inactive monomers (Schwartz, 1994).

Tryptase is the principal protein mediator component of human mast cell granules, accounting for over 20% of the total cellular protein (Schwartz, 1994). MCT is a specific marker of mast cells, allowing for their differentiation from basophils.

Mast cells are found in many tissues but are present in greater numbers along the epithelial linings of the body, such as the skin, respiratory tract and gastrointestinal tract. Mast cells are often located in the proximity of small blood vessels. They are involved in a variety of physiological and pathophysiological states, including acute inflammation, immediate hypersensitivity, delayed-type hypersensitivity, cell growth regulation, defence against neoplasia and the sensation of pain and itch (Liang *et al*, 1998). Mast cells are also implicated in chronic inflammatory states and are involved in neuroimmune interactions (Leon *et al.*, 1994).

Mast cell tryptase is an important inflammatory response mediator. Experiments (mainly performed *in vitro*) suggest it plays important roles in diseases such as asthma, psoriasis, interstitial lung disease, rheumatoid arthritis, gingivitis and periodontitis. Mast cell tryptase has also been implicated in tumorigenesis and angiogenesis, due to its potential to activate pro-urokinase and the matrix metalloproteinase pro-stromelysin. Tryptase-like enzymes

have also been described to take part in the activation and internalisation of pathogenic viruses, such as influenza virus, Sendai virus and human immunodeficiency virus (Pohlig *et al.*, 1996).

Human tryptase is inhibited by small molecular weight substances (e.g. leupeptin and diisopropyl fluorophosphate). Divalent cations, such as calcium, and benzamidine and its derivatives are competitive inhibitors of human mast cell tryptase (Schwartz, 1994). However, human tryptase, unlike most other serine esterases, is not inhibited by classical inhibitors of serine proteases, such as aprotinin and soybean trypsin inhibitor. Endogenous inhibitors that target the catalytic sites of mast cell tryptase have yet to be reported. Human tryptase activity is inhibited by lactoferrin and myeloperoxidase (both neutrophil-derived) and by antithrombin-III, all of which antagonise the glycosaminoglycans (heparin or chondroitin sulfate) that stabilise the MCT tetramer (Alter *et al.*, 1990; Cregar *et al.*, 1999; Elrod *et al.*, 1997).

A leech-derived inhibitor of human tryptase (LDPI) has been previously described. A recombinant form of this Kazal-type protein has been found to inhibit efficiently 2 of the 4 catalytic sites of the tetrameric tryptase (Stubbs *et al.*, 1997; Auerswald *et al.*, 1994; Mühlhahn *et al.*, 1994; Sommerhoff *et al.*, 1994).

Due to the known importance of MCT in mammalian disease and in the allergic response, there is a clear need for highly specific and effective inhibitors of this protein. A novel protein has now been discovered in a tick species that is capable of inhibiting the activity of human mast cell tryptase.

Summary of the invention

According to a first aspect of the present invention there is provided a recombinant protein that exhibits significant sequence homology with the tick-derived protease inhibitor protein (TdPI) sequence given in Figure 1, an active fragment of said protein or a functional equivalent of said protein.

As used herein, the term "significant sequence homology" is meant to include all proteins that share a common function with TdPI and that exhibit common sequence homology or homology between motifs that are present in the polypeptide sequences. "Significant" overall homology refers to 50% or more of the amino acids in the sequence being completely

conserved as identical residues if the homologous protein is aligned with the sequence of TdPI. Preferably, the alignments are obtained using GCG's bestfit command (gap creation penalty = 2.5; gap extension penalty = 0.5)(Genetics-Computer-Group, 1994).

Preferably, the degree of homology is at least 60% across the entire length of the protein. More preferably, the degree of homology is at least 70%, even more preferably 75%, most preferably 80% or more.

Included in this aspect of the invention there is provided a protein comprising the sequence identified herein as tick-derived protease inhibitor protein (TdPI), an active fragment thereof or a functional equivalent thereof. This sequence is given in accompanying Figure 1. This protein was identified as being encoded by a cDNA from a tick salivary gland library. The protein has a molecular weight of approximately 13.5 kDa and appears to belong to the family of Kunitz-type protease inhibitors. The sequence similarity with other members of this family such as aprotinin and inter-alpha-trypsin inhibitor is low, but the putative reactive centre and the position of the cysteines is to some extent conserved.

The term "functional equivalent" is used herein to describe proteins that have an analogous function to the TdPI protein, either in inhibiting tryptase or in possessing one or more epitopes that can be used in the development of vaccines that target proteins that exhibit significant sequence homology with TdPI. The term "functional equivalent" also refers to molecules that are structurally similar to the TdPI protein identified herein or that contain similar or identical tertiary structure. This term also includes protein fragments that retain the ability to inhibit tryptase, preferably human mast cell tryptase.

The analogous function in inhibiting tryptase is preferably directed against the catalytic activity of tryptase, preferably mast cell tryptase, more preferably human mast cell tryptase, is characterised by a Ki of less than 1μM, more preferably 100nM, even more preferably 20nM, even more preferably less than 10nM, most preferably less than 1nM, as assessed using any standard tryptase inhibition assay, such as that described herein (see section entitled "Protease inhibitions assays" in the Examples below).

Alternatively, or in addition to possessing inhibitory activity against tryptase, "functional equivalent" is used herein to describe proteins that contain epitopes which can be used in the development of vaccines against the proteins of the invention. Such functional

equivalents, and also fragments containing suitable epitopes, may be used to develop vaccines directed against blood-feeding parasites, that target members of the TdPI protein family. Functional equivalents may of course be made more or less immunogenic than the corresponding wild type protein or protein fragment in order to suit a desired application. By "wild type" is meant the naturally-occurring genotype that is characteristic of most members of a species. If the proteins are to be used in a vaccination regime to induce host resistance to parasite proteins, then the molecules may be modified so as to enhance their immunogenicity. They will thus be more likely to elicit an immune response in the vaccinated host.

Functional equivalents of the proteins of the invention will include single or multiple amino-acid substitution(s), addition(s), insertion(s) and/or deletion(s) from the wild type protein sequence and substitutions of chemically-modified amino acids that do not affect the function or activity of the protein in an adverse manner. This term is also intended to include natural biological variants (e.g. allelic variants or geographical variations within all the different species from which the wild type proteins are derived).

"Active" fragments are those that either inhibit tryptase, preferably human mast cell tryptase, and/or contain one or more epitopes that can be used in the development of vaccines against the proteins of the present invention. These biological properties are described above.

Preferably, the proteins of this aspect of the invention are derived from blood-feeding ectoparasites, such as mosquitoes or leeches, or from venomous animals such as spiders, scorpions or snakes. More preferably, the proteins are derived from ticks, most preferably Ixodid ticks such as *Rhipicephalus appendiculatus*.

According to a second aspect of the invention there is provided a recombinant protein derived from a blood-feeding arthropod ectoparasite that inhibits tryptase, an active fragment thereof, or a functional equivalent thereof. Preferably, the recombinant protein is derived from a tick, most preferably an Ixodid tick such as *Rhipicephalus appendiculatus*. The activity of these molecules in inhibiting the catalytic activity of tryptase, preferably mast cell tryptase, more preferably human mast cell tryptase, is characterised by a Ki of less than 1μM, more preferably 100nM, more preferably 20nM, even more preferably less than 10nM, most preferably 1nM or less.

Derivatives of the proteins of the above-described aspects of the invention are included as embodiments of the invention. Such derivatives may include an additional protein or polypeptide fused at its amino- or carboxy-terminus or added internally. The purpose of the additional polypeptide may be to aid detection, expression, separation or purification of the protein or may be to lend the protein additional properties as desired. Examples of potential fusion partners include ß-galactosidase, glutathione-S-transferase, luciferase, a polyhistidine tag, a T7 polymerase fragment and a secretion signal peptide.

The proteins of the present invention can be prepared using known techniques of molecular biology and protein chemistry. Protein fragments may be prepared by chemical synthesis, a technique that is especially useful for the generation of short peptides derived from the full length protein sequence, for use as immunogens.

The proteins of the invention may be prepared in recombinant form by expression in a host cell. Such expression methods are well known to those of skill in the art and many are described in detail by Sambrook *et al*, 1989, and Fernandez & Hoeffler, 1998.

A third aspect of the invention provides for the use of the proteins, protein fragments and functional equivalents of the invention to inhibit a tryptase, such as mast cell tryptase, in mammals, thereby to regulate its action and to control its pathological effects. Such molecules may also be used to inhibit trypsin, plasmin and, to a lesser degree, tissue kallikrein.

The invention also includes the use of the above-described proteins, protein fragments and functional equivalents as anti-inflammatory agents. Preferably, these molecules are provided as a pharmaceutical composition including an inert carrier. The protein, protein fragment or functional equivalent may constitute the sole active component of the composition or can form part of a therapeutic package, such as a component of creams for topical administration to insect, snake or scorpion bites, or to skin affected by dermatitis. It may also be used as a carrier molecule for tryptase and tryptase-related compounds, in creams, oils, powders or pills, to provide slow release of the bound components.

The invention also comprises the use of the proteins, protein fragments and functional equivalents of the invention for the quantification of tryptase levels, preferably human mast cell tryptase levels, for example, in blood, nasal lavage fluid, tissues or food products. This

may be as part of a kit that comprises one or more proteins, protein fragments or functional equivalents of the invention, together with means of detection (for example radiolabeled tryptase, antibodies, enzymes such as alkaline phosphatases, peroxidases and luciferases) that allow the accurate quantification of tryptase in the sample to be tested. Such kits may resemble radioimmunoassay or ELISA kits, with the proteins of the invention acting as binding molecules, rather than antibodies directed against tryptase or against tryptase-related molecules. One aspect of the present invention comprises such kits incorporating the molecules of the present invention.

The proteins, protein fragments and functional equivalents of the invention can also be used for the detection of cells carrying tryptase, and in particular for the detection of mast cells. Any technique common to the art may be used in such a detection method and may comprise immunocytochemical and histological techniques, in which the protein, protein fragment or functional equivalent is used in combination with antisera (such as anti-TdPI antisera), or in which the molecule is directly coupled to a label or dye, such as FITC. An entire protein may be used, or simply an active binding fragment in order to detect substrate. In another embodiment, the wild type protein may be fused either genetically or synthetically to another protein such as an alkaline phosphatase, luciferase or peroxidase in order to facilitate its detection. Other methods to detect tryptase-containing cells or samples may involve blotting techniques (Towbin *et al*, 1979), gel retardation, affinity chromatography, or any of the other suitable methods that are used in the art.

The invention also comprises the use of the proteins, protein fragments and functional equivalents of the present invention bound to a support to remove, purify, isolate or extract tryptase, for instance from body tissues, blood or food products. The support may comprise any suitable inert material and includes gels, magnetic and other beads, microspheres, binding columns and resins.

The present invention also includes the use of the proteins, protein fragments and functional equivalents of the invention as tools in the study of inflammation, inflammation-related processes or other physiological processes involving tryptase. These molecules may also be used as tools to study further the characteristics and functions of MCT itself. For example, the molecules may be used for tryptase inhibition or depletion in cell cultures or in inflamed animal tissues, in order to study the importance of tryptase in these systems.

Metazoan parasites, particularly arthropods and helminths, are also sources of infectious diseases and other injurious effects that have major impacts in human and veterinary medicine. Control of arthropod and helminth parasites currently relies primarily on the use of chemicals such as acaricides and antihelmintics. Attempts have been made to use immunological means of control through the use of vaccine technology. There has been some success in identifying certain protective antigens as potential vaccine candidates, but only a few have as yet come to commercial fruition, most notably for the cattle lungworm *Dictyocaulus viviparous* and the cattle tick *Boophilus microplus*. Despite these developments, there is a continuing need for metazoan parasite vaccines and in particular for a vaccine which may be used across a broad range of arthropod and/or helminth genera.

The present invention therefore also provides for the use of the proteins, protein fragments and functional equivalents of the invention as immunogens for use as metazoan parasite vaccines and in particular as protective immunogens in the control of diseases caused by arthropod and other metazoan parasites. Suitable candidates for vaccination include domesticated animals such as cattle, goats, sheep, dogs, cats and other animals which require protection against metazoan parasites, especially ticks. The vaccine may include certain compounds for use as adjuvants. Suitable adjuvants are well known in the art and include oil-in-water emulsion formulations, saponin adjuvants, Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA), cytokines, and other substances that act as immunostimulating agents to enhance the effectiveness of the composition.

According to a still further aspect of the present invention, there is provided a method of vaccinating a mammal against a disease or condition, comprising administering to a mammal a protein, protein fragment or functional equivalent according to the above-described aspects of the invention whose expression is associated with the disease or condition.

A further aspect of the invention provides a method of treating a mammal suffering from a disease or a condition such as asthma, psoriasis, an interstitial lung disease, rheumatoid arthritis, gingivitis, peridontitis, an allergic reaction, cancer or any other tryptase-mediated condition, comprising administering to said mammal a protein, protein fragment or functional equivalent according to the above-described aspects of the invention in a therapeutically-effective amount, optionally in conjunction with a pharmaceutically-acceptable carrier.

According to a further aspect of the present invention there is provided an immunogenic composition comprising a protein, protein fragment or functional equivalent of the above-described aspects of the invention in conjunction with a pharmaceutically-acceptable carrier.

Pharmaceutically-acceptable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes) and inactive virus particles. Such carriers are well known to those of skill in the art. The composition may be used as a vaccine and may thus optionally comprise an immunostimulating agent (adjuvant) for instance an adjuvant as referred to above. According to a further aspect of the invention, there is provided a process for the formulation of a vaccine composition comprising bringing a protein, protein fragment or functional equivalent according to the above-described aspects of the invention into association with a pharmaceutically-acceptable carrier, optionally with an adjuvant.

According to a further aspect of the invention there is provided a nucleic acid molecule comprising a nucleotide sequence encoding a protein, protein fragment or functional equivalent of the above-described aspects of the invention. Such molecules include single- or double-stranded DNA, cDNA and RNA, as well as synthetic nucleic acid species. Preferably, the nucleic acid sequences comprise DNA.

A cDNA encoding TdPI is disclosed herein by way of example and its sequence and the amino acid sequence it encodes are shown in Figure 1 (nucleotides and amino acids are given in their standard one letter abbreviations).

A preferred nucleic acid molecule according to the invention comprises a nucleotide sequence identical to or complementary to the sequence shown in Figure 1, or a sequence that is degenerate or substantially homologous therewith, or which hybridises with this sequence under non-stringent conditions, for instance 6 x SSC/50% formamide at room temperature, and washed under conditions of low stringency, for instance (2 x SSC room temperature or 2 x SSC, 42°C or, more preferably, binding under conditions of higher stringency, e.g. 2 x SSC, 65°C. (SSC = 0.15M NaCl, 0.015M sodium citrate, pH 7.2).

Preferably, said nucleic acid sequences display at least 60% identity to the cDNA encoding TdPI, or DNA sequences of which the translation product (either a partial stretch or the complete translation product) displays at least 60% or more identity with the TdPI sequence, when aligned, preferably using GCG's bestfit command (gap creation penalty = 2.5; gap extension penalty = 0.5) (Genetics Computer Group, 1994).

The invention also includes cloning and expression vectors containing the DNA sequences of this aspect of the invention. Such expression vectors may incorporate the appropriate transcriptional and translational control sequences, for example enhancer elements, promoter-operator regions, termination stop sequences, mRNA stability sequences, start and stop codons or ribosomal binding sites, linked in frame with the nucleic acid molecules of the invention.

Additionally, it may be convenient to cause the recombinant protein to be secreted from certain hosts. Accordingly, further components of such vectors may include nucleic acid sequences encoding secretion signalling and processing sequences.

Vectors according to the invention include plasmids and viruses (including both bacteriophage and eukaryotic viruses), as well as other linear or circular DNA carriers, such as those employing transposable elements or homologous recombination technology. Many such vectors and expression systems are well known and documented in the art (Fernandez & Hoeffler, 1998). Particularly suitable viral vectors include baculovirus-, adenovirus- and vaccinia virus-based vectors.

Suitable hosts for recombinant expression include commonly-used prokaryotic species, such as *E. coli*, or eukaryotic yeasts that can be made to express high levels of recombinant proteins and that can easily be grown in large quantities. Mammalian cell lines grown *in vitro* are also suitable, particularly when using virus-driven expression systems. Another suitable expression system is the baculovirus expression system that involves the use of insect cells as hosts. An expression system may also constitute host cells that have the encoding DNA incorporated into their genome. Proteins, or protein fragments may also be expressed *in vivo*, for example in insect larvae or in mammalian tissues.

A variety of techniques are known and may be used to introduce the vectors according to the present invention into prokaryotic or eukaryotic cells. Suitable transformation or

transfection techniques are well described in the literature (Sambrook *et al*, 1989; Ausubel *et al*., 1991; Spector, Goldman & Leinwald, 1998). In eukaryotic cells, expression systems may either be transient (e.g. episomal) or permanent (chromosomal integration) according to the needs of the system.

Nucleic acid molecules according to the present invention may also be used to create transgenic animals, particularly rodent animals. This may be done locally by modification of somatic cells, or by germ line therapy to incorporate heritable modifications.

The invention also includes transformed or transfected prokaryotic or eukaryotic host cells, or transgenic organisms containing a nucleic acid molecule as defined above.

A further aspect of the invention provides a method for preparing a protein, protein fragment of functional equivalent of the invention, as defined above, which comprises culturing a host cell containing a nucleic acid molecule according to the invention under conditions whereby said protein is expressed and recovering said protein thus produced.

Various aspects and embodiments of the present invention will now be described in more detail by way of example, with particular reference to a protein isolated from the tick, *Rhipicephalus appendiculatus*. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

Brief description of figures

Figure 1 shows the cDNA sequence and inferred amino-acid sequence of TdPI-encoding clone 76-3.

Figure 2 shows a 15% SDS-polyacrylamide gel showing rTdPI, purified by means of metal-affinity chromatography and cation exchange.

Figure 3 shows an alignment of TdPI with Kunitz domains of the bovine colostrum trypsin inhibitor (BovCol; Cechova, 1976), (bovine) aprotinin (Creighton & Charles, 1987), and the rat tissue factor pathway inhibitor (TFPI-2; only the second, factor Xa-inhibiting domain is shown; Enjyoji *et al.*, 1992).

Figure 4 shows a diagram showing the relatively weak inhibitory activity of rTdPI on tissue kallikrein.

Figure 5 shows the activities of plasmin (left) and trypsin (right) in the presence of increasing amounts of rTdPI as determined by measuring peptide release from resorufin-labelled casein.

Figure 6 shows the inhibition of recombinant human tryptase (Promega) with TdPI.

Figure 7 shows a 1.5% agarose gel showing the RT-PCR products obtained with whole-body extracts from larvae (L) and nymphs (N), and with salivary gland extracts from adult, *R. appendiculatus* males and females.

EXAMPLES

Ticks

Ticks were reared according to Jones *et al.*, 1988. All three developmental stages of *R. appendiculatus* were fed on Dunkin Hartley guinea pigs. When not feeding, all ticks were maintained at 21 to 26°C and 85% relative humidity.

cDNA

Clone 76, containing the TdPI cDNA, was one of several clones randomly picked from a *R. appendiculatus* salivary gland expression library in Lambda Zap II (Stratagene), which was constructed with mRNA from ticks that had been feeding on Dunkin Hartley guinea pigs for 2 days (Paesen & Nuttall, 1996). Phagemid was excised *in vivo* and used to generate double-stranded pBluescript SK(-) plasmid in XL1-Blue cells (Short *et al.*, 1988). Plasmid was purified from overnight cultures (Goode & Feinstein, 1992) and alkali-denatured (Mierendorf & Pfeffer, 1987) before sequencing according to Sanger & Coulson, 1975.

The complete sequences of both the plus and minus strand of the 76-3 insert were determined. The forward primer (S1→) (corresponding with nucleotides 209 to 224), reverse primer (←S2) (annealing to nucleotides 255 to 271) and the plasmid-specific T3 (T3→) and T7 (← T7) primers (insert-specific primer sequences, or their annealing sites, are underlined) are shown in Figure 1. P1→ and P2 ← denote the primer sites used in the RT-PCR experiment.

The sequence obtained by N-terminal sequencing of the rTdPI protein is in bold italics in Figure 1. The wave denotes a heparin-binding consensus sequence. The double line indicates a putative glycosylation site. The polyadenylation signal and the polyA-tail are shown in bold letter type. The leucine indicated by the asterisks is a methionine in clones 76, 76-1 and 76-2.

Sequence data were analysed using the GCG sequence analysis software [Genetics-Computer-Group, 1994 #14]. Protein database searches were done at the National Centre for Biotechnology Information (NCBI) using the BLAST network service (Altschul *et al.*, 1990).

Once clone 76 was sequenced, the library was rescreened for additional clones by DNA hybridization of plaque lifts (Sambrook, Fritsch & Maniatis, 1989). The probe used was constructed by random primer labelling of the original cDNA (excised from purified plasmid using *Eco*RI and *Eco*0109I) with digoxygenin (Boehringer Mannheim). Three positive clones were isolated and sequenced.

Recombinant protein expression

Recombinant TdPI (rTdPI) was expressed as a histidine-tagged protein in *Spodoptera frugiperda* ovarian cells (*Sf*21; Invitrogen). The coding region of the TdPI cDNA was amplified by the polymerase chain reaction (PCR), using the forward primer

5'-GCAGGAGCTCGGCACGAG

and the reverse primer

5'-TATGGATCCCAGGTCCAGGCTCTGTTCCG,

thereby adding a *Sac* I site upstream of the start codon, and replacing the stop codon with a *Bam* HI site. The PCR consisted of 20 cycles with a 30-second melting step (95°C), a 30-second primer-annealing step (50°C) and a 30-second extension step (72°C). The PCR product was ligated between the *Sac* I and *Bam* HI sites of the pAC129.1 transfer vector (Livingstone & Jones, 1989), which was modified so that a carboxyterminal Gly-Ile-$(His)_6$ tag was added to the expressed protein. Co-transfection of *Sf*21 cells with the transfer vector and baculovirus (BacPak6) and amplification of recombinant virus was as described

by Kitts & Possee, 1993. rTdPI was expressed in TC100 medium (Gibco BRL) containing 10% foetal bovine serum (Sigma).

Recombinant protein purification

Sixty hours after infection of the *Sf*21 cells, the culture medium was collected and rTdPI was precipitated by addition of $(NH_4)_2SO_4$ (30 g per 100 ml medium). The pellet was redissolved in 50 mM sodium phosphate buffer (pH 8) containing 300 mM NaCl and 10% glycerol. rTdPI was purified using a Ni-NTA agarose (Qiagen) column, mainly according to Janknecht *et al.*, 1991. 50 mM sodium phosphate buffer (pH 6.5) containing 300 mM NaCl and 10% glycerol was used to wash the column. The histidine-tagged protein was eluted using 200 mM imidazole in 75 mM NaH_2PO. Further purification was obtained by low pressure chromatography using the BioLogic system (Bio-Rad) with a HiTrap SP cation exchange column (Pharmacia Biotech). The running buffer was 50 mM Hepes, pH 8, with a linear 0 to 250 mM NaCl gradient over 1 hour; the flow rate was 1 ml/min. Centricon 3 concentrators (Amicon) were used for concentration of the eluants and for buffer exchange. The purified protein was stored at -20 °C in PBS until use. Protein concentration was measured using the Bio-Rad Protein Assay and the Micro BCA Protein Assay (Pierce).

Protein electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was according to Laemmli, 1970.

Figure 2 shows a 15% SDS-polyacrylamide gel showing rTdPI, purified by means of metal-affinity chromatography and cation exchange. The protein in lane A had been treated with PNGase F (the ~35 kDa protein on the gel) prior to electrophoresis. Lane B contains untreated rTdPI. The molecular masses are given in kDa. Lane C contains unreduced rTdPI (no reducing agent in the loading buffer). The higher molecular weight at which unreduced rTdPI runs would normally suggest dimerization through intermolecular disulphide bridges, but mass-spectrometry places the molecular mass at about 13,500 Da, contradicting the formation of dimers.

Asparagine-linked glycosylation was studied by treating rTdPI with N-glycosidase F (PNGase F; New England BioLabs), followed by SDS-PAGE. PNGase F hydrolyses all common types of Asn-glycan chains from glycoproteins (Maley *et al.*, 1989).

Figure 3 shows an alignment of TdPI with Kunitz domains of the bovine colostrum trypsin inhibitor (BovCol; Cechova, 1976), (bovine) aprotinin (Creighton & Charles, 1987), and the rat tissue factor pathway inhibitor (TFPI-2; only the second, factor Xa-inhibiting domain is shown; Enjyoji *et al.*, 1992). The Kunitz domains of the tick anticoagulant peptide TAP (Waxman *et al.*, 1990) and the two domains in ornithodorin (ornith1 and ornith2; Van de Locht *et al.*, 1996) are also included. The alignment of TdPI with the vertebrate Kunitz domains was created using GCG's "pileup" and "prettyplot" commands, choosing relatively low gap and length weights (1 and 0.03, respectively). The alignment was then modified, mainly by introducing extra gaps, so that the TAP and ornithodorin domains could be included. The modification was largely based on the alignment of the latter domains with aprotinin, as reported by Van de Locht *et al.*, 1996. The arrow indicates the P1 residue of the aprotinin binding loop. The asterisks denote the cysteines involved in disulphide-bridge formation in traditional Kunitzdomains.

N-terminal sequencing

The amino-terminal sequence of rTdPI was determined at the MRC Immunochemistry Unit of the Department of Biochemistry of the University of Oxford, according to Matsudaira, 1987. Electroblotted samples were run on an Applied Biosystems 494A 'Procise' protein sequencer (Perkin-Elmer) using an Applied Biosystems 'Mini-Blott' cartridge.

Mass Spectrometry

ESI-MS was performed on a VG BioQ triple quadrapole atmospheric pressure mass spectrometer equipped with an electrospray interface operating in positive ion mode. The instrument was calibrated with horse heart myoglobin (7 pmol/µl; average molecular mass 16,951.48 Da).

Protease inhibition assays

Elastase (type I, from porcine pancreas), α-chymotrypsin, trypsin, thrombin, plasmin, tissue kallikrein, plasma kallikrein, urokinase, aprotinin, n-succinyl-Ala-Ala-Ala-p-nitroanilide, Gly-Arg-p-nitroanilide, n-α-benzoyl-DL-Arg-p-nitroanilide and n-benzoyl-Pro-Phe-Arg-p-nitroanilide were purchased from Sigma. Factor Xa and recombinant human tryptase were from Promega and resorufin-labelled casein, soybean trypsin inhibitor, Chromozym TH and Chromozym X were obtained from Boehringer Mannheim.

Tryptase activity was measured in 96-well microplates, using n-α-benzoyl-DL-Arg-p-nitroanilide as chromogenic substrate and 50 mM HEPES pH 7.6, containing 120 mM NaCl, as reaction buffer. 50 µl buffer containing 1 µl of the tryptase stock (200 µg/ml) was combined with 50 µl of inhibitor solution (various concentrations). After a 45-minute incubation period at 37 °C, 50 µl of 3 mM substrate solution was added and the increase in absorbance at 405 nm was measured using a Titertek Multiskan Plus MKII plate reader (ICN).

Other proteases were preincubated with various amounts of protease inhibitor in a total volume of 100 µl protease buffer (20 minutes; 37 °C). The residual protease activity was determined by adding the appropriate substrates (in 900 µl protease buffer) and measuring the degree of digestion. Trypsin, α-chymotrypsin, and elastase activities were measured in protease buffer A (0.1 M Tris.HCl, 10 % glycerol, 10 mM $CaCl_2$, pH 8); plasmin, urokinase, kallikrein, α-thrombin and factor Xa activities were determined in protease buffer B (50 mM Tris.HCl, 0.1 mg/ml bovine serum albumin, 150 mM NaCl, 1 mM $CaCl_2$, pH 8), as described by Nakamura *et al.*, 1987. Resorufin-labelled casein was used as a substrate for trypsin, α-chymotrypsin and plasmin, and the amount of released peptide was measured to determine protease activity (Twining, 1984). p-Nitroanilide (pNA)-substrates were used for elastase, kallikrein, urokinase, α-thrombin and factor Xa activities (n-succinyl-Ala-Ala-Ala-pNA, n-benzoyl-Pro-Phe-Arg-pNA, Gly-Arg-pNA, Chromozym TH, and Chromozym X, respectively); protease activity was measured by determining the increase in absorbance at 410 nm.

Figure 4 shows a diagram showing the relatively weak inhibitory activity of rTdPI on tissue kallikrein. The absorbance at 410 nm is shown at different time points after addition of

30μg substrate (n-benzoyl-Pro-Phe-Arg-pNA) to kallikrein/antiprotease samples. 0.5 u/ml tissue kallikrein was used per sample (1 ml final volume). The full line (♦—♦) denotes kallikrein activity in the absence of protease inhibitor. Aprotinin used at a concentration of 0.75μM completely inhibits kallikrein activity (●----●). A ten times higher concentration of rTdPI [7.5 μM (O----O)] barely inhibits 50% of the kallikrein activity. Other concentrations of rTdPI used in the experiment were 3.75 μM (Δ----Δ) and 0.75 μM (□---□).

Figure 5 shows the activities of plasmin (left) and trypsin (right) in the presence of increasing amounts of rTdPI as determined by measuring peptide release from resorufin-labelled casein. The peptide release in the absence of inhibitor was set to be 100%, hydrolysis in the absence of protease corresponds with 0% activity. The values for rTdPI are denoted by the open circles. To calculate the micromolar concentration of rTdPI monomers from the mg/ml data obtained with the protein assay, both the calculated molecular mass of 12 kDa (O----O ; assuming no binding of Coomassie blue to the carbohydrate fraction of the glycoprotein) and the (average) molecular mass as determined by mass-spectrometry (13.5 kDa; O—O) were used. The concentrations corresponding with a 50% plasmin inhibition are 0.097 μM for aprotinin (Δ—Δ), 0.23 μM for soybean trypsin inhibitor (■—■), 0.32 μM (O—O) and 0.43 μM (O----O) for rTdPI monomers. The values for 50% trypsin inhibition are 0.024 μM (Δ—Δ), 0.026 μM (■—■), 0.033 μM (O—O) and 0.044 μM (O----O).

Figure 6 shows the inhibition of recombinant human tryptase (Promega) with TdPI. Preincubation of recombinant human tryptase with increasing amounts of rTdPI quickly reduces the catalytic activity to about 33% of the activity in the absence of inhibitor (V_0 : the velocity of substrate turnover measured without tryptase present; Vi : the velocity with inhibitor added).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Salivary glands were excised from unfed adult ticks, and from adult ticks that had been feeding on guinea pigs for 2, 4 and 6 days. Each tissue sample consisted of 15 pairs of glands. Total RNA was isolated from these glands using the RN*Ace* Total Pure extraction kit (Bioline Ltd) and 1/30 of the amount obtained (the equivalent of one gland) was used as a template for RT-PCR (35 cycles), utilizing the Titan one tube RT-PCR system

(Boehringer Mannheim). RT-PCR was also carried out on pooled RNA from gut, gonads, accessory sex glands and malpighian tubules, taken from 2-days fed adult ticks. Whole-body homogenates of 3 days-fed larvae and 3 days-fed nymphs were submitted to the same procedure; the amount of RNA used per PCR reaction corresponded with the extract from 1 nymph or 2 larvae. The primer sequences (P1 and P2) are underlined in Figure 1. To check whether the RT-PCR products were specifically derived from TdPI mRNA, their sizes were compared to the size of a marker that was obtained by PCR-amplification of the original plasmid DNA, using the same primers.

Figure 7 shows a 1.5% agarose gel showing the RT-PCR products obtained with whole-body extracts from larvae (L) and nymphs (N), and with salivary gland extracts from adult, *R. appendiculatus* males and females. The numbers correspond with different time points of the adult feeding stage; 0 denotes samples taken from unfed ticks; 2, 4 and 6 indicate 2, 4 and 6 days fed ticks, respectively. Lane M shows as a the molecular weight marker tee PCR product obtained with the same set of primers (Fig. 1), but using the TdPI cDNA as a template, instead of RNA.

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Claims

1. A recombinant protein that exhibits significant sequence homology with the tick-derived protease inhibitor protein (TdPI) sequence given in Figure 1, an active fragment of said protein or a functional equivalent of said protein.

2. A recombinant protein, protein fragment or functional equivalent according to claim 1, that functions as an inhibitor of tryptase, preferably of human mast cell tryptase.

3. A recombinant protein, protein fragment or functional equivalent according to either of claims 1-2 that contains one or more epitopes that can be used in the development of vaccines that target proteins that exhibit significant sequence homology with TdPI.

4. A recombinant protein or protein fragment according to claim 1, wherein said sequence homology is defined as 50% or more of the amino acids in the sequence being completely conserved as identical residues if the protein is aligned with the sequence of Figure 1, the alignments being obtained using GCG's bestfit command (gap creation penalty = 2.5; gap extension penalty = 0.5).

5. A recombinant protein or protein fragment according to claim 4, wherein said sequence homology is 60% or more.

6. A recombinant protein or protein fragment according to claim 5, wherein said sequence homology is 75% or more.

7. A recombinant protein or protein fragment according to any one of claims 1-6 comprising the TdPI sequence.

8. A recombinant protein derived from a blood-feeding arthropod ectoparasite that inhibits tryptase, or an active fragment of said protein or a functional equivalent of said protein.

9. A recombinant protein, protein fragment or functional equivalent according to claim 8, that functions as an inhibitor of tryptase, preferably of human mast cell tryptase.

10. A recombinant protein, protein fragment or functional equivalent according to claim 8 or claim 9 that contains one or more epitopes that can be used in the development of vaccines that target proteins that exhibit significant sequence homology with TdPI.

11. A recombinant protein or protein fragment according to any one of claims 1-10 that inhibits tryptase with a Ki of less than 1×10^{-6} M, preferably less than 1×10^{-7} M, more preferably less than 2×10^{-8} M, most preferably less than 1×10^{-9} M.

12. A recombinant protein, protein fragment or functional equivalent according to any one of the claims 1-11 that inhibits catalytic tryptase activity.

13. A recombinant protein, protein fragment or functional equivalent according to any one of claims 1-12 which inhibits mast cell tryptase, preferably human mast cell tryptase.

14. A recombinant protein, protein fragment or functional equivalent according to any one of the preceding claims, ~~that~~ is derived from a tick.

15. A recombinant protein, protein fragment or functional equivalent according to claim 14, that is derived from the tick *Rhipicephalus appendiculatus*.

16. A recombinant protein, protein fragment or functional equivalent according to any one of the preceding claims that has been genetically or chemically fused to one or more peptides or polypeptides.

17. A recombinant protein, protein fragment or functional equivalent according to any one of the preceding claims that is bound to a support, such as a resin.

18. A pharmaceutical composition comprising a recombinant protein, protein fragment or functional equivalent according to any one of claims 1-17, in conjunction with a pharmaceutically-acceptable carrier.

19. A vaccine composition comprising a recombinant protein, protein fragment or functional equivalent according to any one of claims 1-15, optionally in conjunction with an adjuvant.

20. A process for the formulation of a pharmaceutical composition according to claim 19 comprising bringing a recombinant protein, protein fragment or functional equivalent

according to any one of claims 1-15 into association with a pharmaceutically-acceptable carrier.

21. A recombinant protein, protein fragment or functional equivalent according to any one of claims 1 to 15 for use as a pharmaceutical.

22. A method for the prevention or treatment of a disease in a subject, comprising administering to said subject an effective dose of a composition according to claim 18 or claim 19.

23. A nucleic acid molecule encoding a recombinant protein, protein fragment or functional equivalent according to any one of claims 1-16.

24. A nucleic acid molecule: having the sequence given in Figure 1; which hybridises with said nucleotide sequence under stringent hybridisation conditions; or which encodes on expression a recombinant protein, protein fragment or functional equivalent as defined in any one of claims 1-16.

25. A vector comprising a nucleic acid according to claim 23 or claim 24.

26. The vector of claim 25 that is virus-based.

27. A host cell transformed or transfected with the vector of claim 25 or claim 26.

28. A transgenic animal that has been transformed by a nucleic acid molecule according to claim 23 or claim 24.

29. A method of preparing a recombinant protein, protein fragment or functional equivalent according to any one of claims 1 to 16, comprising expressing a vector according to claim 25 or claim 26 in a host cell and culturing said host cell under conditions where said recombinant protein, protein fragment or functional equivalent is expressed, and recovering said recombinant protein, protein fragment or functional equivalent thus produced.

30. Use of a recombinant protein, protein fragment or functional equivalent according to any one of claims 1-17 for: the detection or quantification of tryptase; for the depletion or removal of tryptase from a food product or from a cell culture; as an anti-tryptase agent; or as an anti-inflammatory drug.

31. Use of a recombinant protein, protein fragment or functional equivalent according to any one of claims 1 to 16 in the manufacture of a medicament for the treatment of inflammation in humans or animals.

32. A method of vaccinating a mammal against a disease, or of treating a mammal suffering from a disease, comprising administering a recombinant protein, protein fragment or functional equivalent according to any one of claims 1 to 16 to a said mammal.

33. Use of a protein or protein fragment selected from the group consisting of bovine colostrum trypsin inhibitor, the rat tissue factor pathway inhibitor (TFPI-2), the Kunitz domain of the tick anticoagulant peptide TAP and the two domains in ornithodorin as a tryptase inhibitor.

TENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P022288WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/02791	19/07/2000	19/07/1999
International Patent Classification (IPC) or national classification and IPC C12N15/15		
Applicant EVOLUTEC LTD et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
19/02/2001	22.10.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Mundel, C Telephone No. +49 89 2399 7314

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/02791

I. Basis of the rep rt

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-21 as originally filed

Claims, No.:

1-33 as originally filed

Drawings, sheets:

1/6-6/6 as originally filed

Sequence listing part of the description, pages:

2-5 (phoenix), filed with the letter of 10.10.00

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	4-7, 16-17, 19, 26, 28 and 30
	No:	Claims	1-3, 8-15, 18, 20-25, 27, 29 and 31-32
Inventive step (IS)	Yes:	Claims	7, 19 and 30
	No:	Claims	1-6, 8-18, 20-29 and 31-32
Industrial applicability (IA)	Yes:	Claims	1-21, 23-29 and 31
	No:	Claims	22, 30 and 32 (see Citations and explanations)

2. Citations and explanations
   see separate sheet

R Item II

Priority

The priority document of the present application was not available at the time where this International Preliminary Examination Report (IPER) has been drafted. The present analysis is based on the hypothesis that all the claims have a priority right corresponding to the date of filing of the priority document (19.07.99).

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive st p or industrial applicability; citations and explanations supporting such stat ment

1. The present application refers to a tick-derived protease inhibitor protein (TdPI) which inhibits mast cell tryptase activity. The application also refers to a nucleic acid encoding said protein, vectors and host cells comprising said nucleic acid, pharmaceutical compositions and methods of treatment implying the use of TdPI.

2. **Reference is made to the following documents :**

   D1: ELROD, K.C. ET AL.: 'Lactoferrin, a potent tryptase inhibitor, abolishes late-phase airway responses in allergic sheep.' AM. J. RESPIRATORY CRITICAL CARE MEDICINE, vol. 156, 1997, pages 375-381.

   D2: KATUNUMA, N.: 'New biological functions of intracellular proteases and their endogenous inhibitors as bioreactants.' ADV. ENZYME REGUL., vol. 30, 1990, pages 377-392.

   D3: WO 95 03333 A (UCP GEN PHARMA AG ;FRITZ HANS (DE); SOMMERHOFF CHRISTIAN P (DE)) 2 February 1995 (1995-02-02)

   D4: WO 96 08275 A (BAYER AG) 21 March 1996 (1996-03-21)

3. **Lack of novelty; article 33(2) PCT.**

   3.1 The attention of the applicant is drawn to the fact that each tryptase inhibitor can be considered as a "functional equivalent" of the TdPI protein of the present application.

Moreover, the attention of the applicant is drawn to the fact that claims directed to a protein have been considered as not characterized by any valid technical feature if the protein is characterized by :

- its origin since, once isolated, the origin of a protein can only be determined by reference to its specific amino acid sequence.
- the result to be achieved by said protein since, according to the PCT Gazette of the 29.10.98 "PCT International Preliminary Examination Guidelines", Chapter III-4.7 : "The area defined by the claims must be as precise as the invention allows. As a general rule, claims which attempt to define the invention, or a feature thereof, by a result to be achieved should be objected to".

3.2 The proteins disclosed in the documents D1 (lactoferrin), D2 (trypstatin and A4-inhibitor), D3 (Leech-derived tryptase inhibitor : LDTI) and D4 (secretory leukocyte protease inhibitor : SLPI), being tryptase inhibitors, can be considered as "functional equivalents" of the protein having the sequence disclosed in Fig. 1 of the present application. Therefore and for the reasons mentioned above (see point V-3.1), claims 1-3 can not be considered as new in the sense of article 33(2) PCT.

Moreover, the document D3 claims the nucleotide sequence encoding the LDTI protein or fragments thereof, vectors containing said nucleotide sequence, host cells transformed with such vectors, pharmaceutical compositions containing LDTI and its use in diagnosis and therapy (Abstract and claims). Therefore, claims 18, 20-25, 27, 29 and 31-32 can not be considered as novel in the sense of article 33(2) PCT.

3.3 The proteins of claims 8-15 are only characterized by their origin and/or the result to be achieved by said proteins. For the reasons mentioned above (see point V-3.1), said proteins are not characterized by any technical feature and thus, the subject-matter of claims 8-15 can not be considered as novel in the sense of article 33(2) PCT.

3.4 The subject-matter of claims 4-7, 16-17, 19, 26, 28 and 30 has never been disclosed in the documents cited in the International Search Report. Therefore, claims 4-7, 16-17, 19, 26, 28 and 30 are considered as novel in the sense of article 33(2) PCT.

4. Lack of inventive step; article 33(3) PCT.

4.1 The proteins claimed in claims 4-6 of the present application should present some similarities with the protein having the sequence shown in Fig. 1 of the present application. However, the attention of the applicant is drawn to the fact that these proteins would not necessarily have the same function as the TdPI protein of the present application. Therefore, inventive step of claims 4-6 can not be recognized (article 33(3) PCT).

4.2 The proteins of claims 1-3 and 8-15 and the nucleic acid of claims 23 and 24 being not new, the generation of fusion proteins (claim 16), the binding of the protein to a support (claim 17), the use of a well-known virus-based vector (claim 26) or the generation of a transgenic animal (claim 28) using technics well-known in the art can not be considered as inventive in the sense of article 33(3) PCT.

4.3 The subject-matter of claims 7, 19 and 30 has never been disclosed or suggested in the documents cited in the International Search Report. Therefore, claims 7, 19 and 30 ave to be considered as inventive in the sense of article 33(3) PCT.

5. Industrial applicability; article 33(4) PCT.

Claims 22 and 32 (completely) and 30 (partially) refer to methods of treatment of the human or animal body.
For the assessment of the present claims 22, 30 and 32 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may

allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Due: 30.9.01

PATENT COOPERATION TREATY

→ HRG flan fm

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
MERCER, Christopher Paul
CARPMAELS & RANSFORD
43 Bloomsbury Square
London WC1A 2RA
GRANDE BRETAGNE

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	30.07.2001
Applicant's or agent's file reference: P022288WO	**REPLY DUE** **within 2 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/02791	19/07/2000	19/07/1999

International Patent Classification (IPC) or both national classification and IPC
C12N15/15

Applicant
EVOLUTEC LTD et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☒	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☐	Certain defects in the international application
VIII	☐	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 19/11/2001.

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer / Examiner
Mundel, C

Formalities officer (incl. extension of time limits)
CLEERE, C
Telephone No. +49 89 2399 8061

I. Basis f the opinion

1. With regard to the **elements** of the international application (Replacement *sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed"*):

Description, pages:

1-21 as originally filed

Claims, No.:

1-33 as originally filed

Drawings, sheets:

1/6-6/6 as originally filed

Sequence listing part of the description, pages:

2-5 (phoenix), filed with the letter of 10.10.00

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Pri rity

1. ☐ This opinion has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-3, 18, 20-25, 27, 29 and 31-32 (NO)
Inventive step (IS)	Claims	1-6, 8-18, 20-29 and 31-32 (NO)
Industrial applicability (IA)	Claims	22, 30 and 32 (see Citations and explanations)

2. Citations and explanations
   see separate sheet

R Item II
Priority

The priority document of the present application was not available at the time where this preliminary opinion has been drafted. The present analysis is based on the hypothesis that all the claims have a priority right corresponding to the date of filing of the priority document (19.07.99).

R Item V
R asoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive st p or industrial applicability; citations and explanations supporting such stat m nt

1. The present application refers to a tick-derived protease inhibitor protein (TdPI) which inhibits mast cell tryptase activity. The application also refers to a nucleic acid encoding said protein, vectors and host cells comprising said nucleic acid, pharmaceutical compositions and methods of treatment implying the use of TdPI.

2. **Reference is made to the following documents :**

D1: ELROD, K.C. ET AL.: 'Lactoferrin, a potent tryptase inhibitor, abolishes late-phase airway responses in allergic sheep.' AM. J. RESPIRATORY CRITICAL CARE MEDICINE, vol. 156, 1997, pages 375-381.

D2: KATUNUMA, N.: 'New biological functions of intracellular proteases and their endogenous inhibitors as bioreactants.' ADV. ENZYME REGUL., vol. 30, 1990, pages 377-392.

D3: WO 95 03333 A (UCP GEN PHARMA AG ;FRITZ HANS (DE); SOMMERHOFF CHRISTIAN P (DE)) 2 February 1995 (1995-02-02)

D4: WO 96 08275 A (BAYER AG) 21 March 1996 (1996-03-21)

3. **Lack of novelty; article 33(2) PCT.**

3.1 The attention of the applicant is drawn to the fact that each tryptase inhibitor can be considered as a "functional equivalent" of the TdPI protein of the present application.

Moreover, the attention of the applicant is drawn to the fact that claims directed to a protein have been considered as not characterized by any valid technical feature if the protein is characterized by :

- its origin since, once isolated, the origin of a protein can only be determined by reference to its specific amino acid sequence.
- the result to be achieved by said protein since, according to the PCT Gazette of the 29.10.98 "PCT International Preliminary Examination Guidelines", Chapter III-4.7 : "The area defined by the claims must be as precise as the invention allows. As a general rule, claims which attempt to define the invention, or a feature thereof, by a result to be achieved should be objected to".

3.2 The proteins disclosed in the documents D1 (lactoferrin), D2 (trypstatin and A4-inhibitor), D3 (Leech-derived tryptase inhibitor : LDTI) and D4 (secretory leukocyte protease inhibitor : SLPI), being tryptase inhibitors, can be considered as "functional equivalents" of the protein having the sequence disclosed in Fig. 1 of the present application. Therefore and for the reasons mentioned above (see point V-3.1), claims 1-3 can not be considered as new in the sense of article 33(2) PCT.

Moreover, the document D3 claims the nucleotide sequence encoding the LDTI protein or fragments thereof, vectors containing said nucleotide sequence, host cells transformed with such vectors, pharmaceutical compositions containing LDTI and its use in diagnosis and therapy (Abstract and claims). Therefore, claims 18, 20-25, 27, 29 and 31-32 can not be considered as novel in the sense of article 33(2) PCT.

3.3 The proteins of claims 8-15 are only characterized by their origin and/or the result to be achieved by said proteins. For the reasons mentioned above (see point V-3.1), said proteins are not characterized by any technical feature and thus, the subject-matter of claims 8-15 can not be considered as novel in the sense of article 33(2) PCT.

4. Lack of inventive st p; articl 33(3) PCT.

4.1 The proteins claimed in claims 4-6 of the present application should present some similarities with the protein having the sequence shown in Fig. 1 of the present application. However, the attention of the applicant is drawn to the fact that these proteins would not necessarily have the same function as the TdPI protein of the present application. Therefore, inventive step of claims 4-6 can not be recognized (article 33(3) PCT).

4.2 The proteins of claims 1-3 and 8-15 and the nucleic acid of claims 23 and 24 being not new, the generation of fusion proteins (claim 16), the binding of the protein to a support (claim 17), the use of a well-known virus-based vector (claim 26) or the generation of a transgenic animal (claim 28) using technics well-known in the art can not be considered as inventive in the sense of article 33(3) PCT.

5. Industrial applicability; article 33(4) PCT.

Claims 22 and 32 (completely) and 30 (partially) refer to methods of treatment of the human or animal body.
For the assessment of the present claims 22, 30 and 32 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

6. The applicant is requested to file new claims which take account of the above comments.
The applicant is invited to specifically indicate which amendments have been made and where a basis may be found for these amendments in the application as originally filed (Rule 66.8(a) PCT). Failure to comply with this invitation will result in the amendments not being examined.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

MERCER, Christopher Paul
CARPMAELS & RANSFORD
43 Bloomsbury Square
London WC1A 2RA
GRANDE BRETAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	22.10.2001
Applicant's or agent's file reference P022288WO	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/02791	19/07/2000	19/07/1999

Applicant
EVOLUTEC LTD et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Hingel, W Tel.+49 89 2399-8717

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P022288WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/02791	19/07/2000	19/07/1999
International Patent Classification (IPC) or national classification and IPC C12N15/15		
Applicant EVOLUTEC LTD et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
19/02/2001	22.10.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Mundel, C Telephone No. +49 89 2399 7314

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/02791

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-21 as originally filed

Claims, No.:

1-33 as originally filed

Drawings, sheets:

1/6-6/6 as originally filed

Sequence listing part of the description, pages:

2-5 (phoenix), filed with the letter of 10.10.00

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   s e separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	4-7, 16-17, 19, 26, 28 and 30
	No:	Claims	1-3, 8-15, 18, 20-25, 27, 29 and 31-32
Inventive step (IS)	Yes:	Claims	7, 19 and 30
	No:	Claims	1-6, 8-18, 20-29 and 31-32
Industrial applicability (IA)	Yes:	Claims	1-21, 23-29 and 31
	No:	Claims	22, 30 and 32 (see Citations and explanations)

2. Citations and explanations
   see separate sheet

R Item II

Priority

The priority document of the present application was not available at the time where this International Preliminary Examination Report (IPER) has been drafted. The present analysis is based on the hypothesis that all the claims have a priority right corresponding to the date of filing of the priority document (19.07.99).

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The present application refers to a tick-derived protease inhibitor protein (TdPI) which inhibits mast cell tryptase activity. The application also refers to a nucleic acid encoding said protein, vectors and host cells comprising said nucleic acid, pharmaceutical compositions and methods of treatment implying the use of TdPI.

2. **Reference is made to the following documents :**

   D1: ELROD, K.C. ET AL.: 'Lactoferrin, a potent tryptase inhibitor, abolishes late-phase airway responses in allergic sheep.' AM. J. RESPIRATORY CRITICAL CARE MEDICINE, vol. 156, 1997, pages 375-381.

   D2: KATUNUMA, N.: 'New biological functions of intracellular proteases and their endogenous inhibitors as bioreactants.' ADV. ENZYME REGUL., vol. 30, 1990, pages 377-392.

   D3: WO 95 03333 A (UCP GEN PHARMA AG ;FRITZ HANS (DE); SOMMERHOFF CHRISTIAN P (DE)) 2 February 1995 (1995-02-02)

   D4: WO 96 08275 A (BAYER AG) 21 March 1996 (1996-03-21)

3. **Lack of novelty; article 33(2) PCT.**

   3.1 The attention of the applicant is drawn to the fact that each tryptase inhibitor can be considered as a "functional equivalent" of the TdPI protein of the present application.

Moreover, the attention of the applicant is drawn to the fact that claims directed to a protein have been considered as not characterized by any valid technical feature if the protein is characterized by :

- its origin since, once isolated, the origin of a protein can only be determined by reference to its specific amino acid sequence.
- the result to be achieved by said protein since, according to the PCT Gazette of the 29.10.98 "PCT International Preliminary Examination Guidelines", Chapter III-4.7 : "The area defined by the claims must be as precise as the invention allows. As a general rule, claims which attempt to define the invention, or a feature thereof, by a result to be achieved should be objected to".

3.2 The proteins disclosed in the documents D1 (lactoferrin), D2 (trypstatin and A4-inhibitor), D3 (Leech-derived tryptase inhibitor : LDTI) and D4 (secretory leukocyte protease inhibitor : SLPI), being tryptase inhibitors, can be considered as "functional equivalents" of the protein having the sequence disclosed in Fig. 1 of the present application. Therefore and for the reasons mentioned above (see point V-3.1), claims 1-3 can not be considered as new in the sense of article 33(2) PCT.

Moreover, the document D3 claims the nucleotide sequence encoding the LDTI protein or fragments thereof, vectors containing said nucleotide sequence, host cells transformed with such vectors, pharmaceutical compositions containing LDTI and its use in diagnosis and therapy (Abstract and claims). Therefore, claims 18, 20-25, 27, 29 and 31-32 can not be considered as novel in the sense of article 33(2) PCT.

3.3 The proteins of claims 8-15 are only characterized by their origin and/or the result to be achieved by said proteins. For the reasons mentioned above (see point V-3.1), said proteins are not characterized by any technical feature and thus, the subject-matter of claims 8-15 can not be considered as novel in the sense of article 33(2) PCT.

3.4 The subject-matter of claims 4-7, 16-17, 19, 26, 28 and 30 has never been disclosed in the documents cited in the International Search Report. Therefore, claims 4-7, 16-17, 19, 26, 28 and 30 are considered as novel in the sense of article 33(2) PCT.

4. Lack of inventive step; article 33(3) PCT.

4.1 The proteins claimed in claims 4-6 of the present application should present some similarities with the protein having the sequence shown in Fig. 1 of the present application. However, the attention of the applicant is drawn to the fact that these proteins would not necessarily have the same function as the TdPI protein of the present application. Therefore, inventive step of claims 4-6 can not be recognized (article 33(3) PCT).

4.2 The proteins of claims 1-3 and 8-15 and the nucleic acid of claims 23 and 24 being not new, the generation of fusion proteins (claim 16), the binding of the protein to a support (claim 17), the use of a well-known virus-based vector (claim 26) or the generation of a transgenic animal (claim 28) using technics well-known in the art can not be considered as inventive in the sense of article 33(3) PCT.

4.3 The subject-matter of claims 7, 19 and 30 has never been disclosed or suggested in the documents cited in the International Search Report. Therefore, claims 7, 19 and 30 ave to be considered as inventive in the sense of article 33(3) PCT.

5. Industrial applicability; article 33(4) PCT.

Claims 22 and 32 (completely) and 30 (partially) refer to methods of treatment of the human or animal body.
For the assessment of the present claims 22, 30 and 32 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may

allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

PATENT COOPERATION TREATY

PCT

as

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
P022288WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/ 02791	19/07/2000	19/07/1999
Applicant		
EVOLUTEC LTD		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

TRYPTASE INHIBITOR

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

Intern. Application No
PCT/GB 00/02791

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/15 C12N15/62 C07K14/435 A61K38/17 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal, PAJ, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 03333 A (UCP GEN PHARMA AG ;FRITZ HANS (DE); SOMMERHOFF CHRISTIAN P (DE)) 2 February 1995 (1995-02-02) page 1, paragraph 1 -page 3, paragraph 3 ---	1-32
A	WO 96 08275 A (BAYER AG) 21 March 1996 (1996-03-21) page 2, paragraph 2 - paragraph 3 ---	1-32
A	ELROD, K.C. ET AL.: "Lactoferrin, a potent tryptase inhibitor, abolishes late-phase airway responses in allergic sheep." AM. J. RESPIRATORY CRITICAL CARE MEDICINE, vol. 156, 1997, pages 375-381, XP000971405 page 375, column 2, paragraph 1 -page 376, column 1, paragraph 3 ---	1-32

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 2001	17 04 2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Mata Vicente, T.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter. [redacted] pplication No
PCT/GB 00/02791

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KATUNUMA, N.: "New biological functions of intracellular proteases and their endogenous inhibitors as bioreactants." ADV. ENZYME REGUL., vol. 30, 1990, pages 377-392, XP000971432 page 381, paragraph 2 page 381, last paragraph page 383, line 2 - line 3	1-33
A	RICE ET AL: "Inhibitors of Tryptase for the treatment of Mast Cell-Mediated Diseases" CURRENT PHARMACEUTICAL DESIGN,NL,BENTHAM SCIENCE PUBLISHERS, SCHIPHOL, vol. 4, no. 5, 1998, pages 381-396, XP002108322 ISSN: 1381-6128 page 394, paragraph 2	18-22, 30-32
A	PAESEN ET AL: "Histamine - binding proteins in tick saliva" ANNUAL MEETING OF PROFESSIONAL RESEARCH SCIENTISTS. EXPERIMENTAL BIOLOGY,XX,XX, vol. 12, 1998, page A1001 XP002099797 the whole document	30-32
A	WO 93 14121 A (NOVONORDISK AS) 22 July 1993 (1993-07-22) page 12, line 11 -page 13, line 10	33
A	WO 90 14841 A (CALIFORNIA BIOTECHNOLOGY INC) 13 December 1990 (1990-12-13) page 24, line 25 -page 25, line 11 page 28, line 16 - line 25 page 64, line 31 - line 33	33
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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 22 and 32, in part claim 30 and, as far as an "in vivo" method is concerned, claim 33 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although as far as an "in vivo" method is concerned claim 30 is directed in part to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 00/02791

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

As a result of the prior review under R. 40.2(e) PCT, part of the additional fees are to be refunded.

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[X] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information [redacted] patent family members

Inter[redacted]pplication No
PCT/GB 00/02791

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